INTERACTION OF HUMAN SERUM ALBUMIN WITH THE ELECTROPHILIC METABOLITE 1-O-GEMFIBROZIL-β-D-GLUCURONIDE

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ABSTRACT:

Acyl glucuronides are electrophilic metabolites that are readily hydrolyzed, undergo intramolecular rearrangement, and bind covalently to endogenous proteins. Gemfibrozil is a fibrate lipid-lowering agent that is extensively metabolized to an acyl glucuronide conjugate in humans. The aim of this study was to examine the interactions of 1-O-gemfibrozil-β-D-glucuronide with human serum albumin. The degradation of 1-O-gemfibrozil-β-D-glucuronide (−200 μM) was examined in vitro during incubations at 37°C with phosphate buffer (pH 7.4 or 9.0), solutions of human serum albumin (pH 7.4), or fresh human plasma (pH 7.4). The effects of diazepam, oxyphenbutazone, and gemfibrozil on the degradation of 1-O-gemfibrozil-β-D-glucuronide, and its reversible binding to albumin were also studied. A pilot in vivo study was performed on two patient volunteers administered 1 g/day po gemfibrozil. 1-O-Gemfibrozil-β-D-glucuronide was unstable, with degradation half-lives in buffer of 4.1 hr and 44 hr at pH 9.0 and 7.4, respectively; and 8.5 hr and 5.5 hr in pH 7.4 solutions of human serum albumin or fresh plasma, respectively. Degradation was dependent on pH and the presence of albumin, which seemed to accelerate the intramolecular rearrangement and hydrolysis of the conjugate. 1-O-Gemfibrozil-β-D-glucuronide was highly reversibly bound to albumin, with a mean unbound fraction of 0.028, and its degradation seemed to be related to the degree of reversible binding. Hydrolysis and covalent binding were associated with the site II binding domain on albumin, because only diazepam inhibited these reactions. However, intramolecular rearrangement was increased when binding to the site I domain was inhibited. Covalent binding was also detected in vivo to human plasma proteins. The half-life of the gemfibrozil-protein adducts was 2.5–3 days. Albumin plays an important role in the disposition of acyl glucuronides by acting as: i) a transporter protein; ii) a potential catalyst for their degradation and, therefore, clearance; and iii) a target for covalent adduct formation.

Gemfibrozil (fig. 1) is a fibrate hypolipidemic agent recommended for the clinical management of hyperlipidemias involving raised triglycerides, and is clinically effective in lowering the incidence of coronary heart disease (1, 2). Its elimination in humans involves both oxidative metabolism and conjugation of the carboxylic acid function with glucuronic acid to form the acyl glucuronide conjugate, 1-O-gemfibrozil-β-D-glucuronide (fig. 1) (3, 4). Gemfibrozil glucuronide is excreted in both urine and bile, and up to 50% of a dose of gemfibrozil has been recovered in urine as the acyl glucuronide conjugate in humans (3).

It is now well established that acyl glucuronides are chemically reactive, electrophilic species that undergo nonenzymic nucleophilic substitution reactions with: i) free hydroxyl anions, resulting in hydrolysis of the conjugate and regeneration of the parent acid; ii) the hydroxyl groups on the glucuronic acid moiety, resulting in intramolecular migration of the xenobiotic moiety from the 1-O-β position to the 2-, 3-, and 4-positions of the glucuronic acid ring; and iii) nucleophilic groups on proteins resulting in covalent binding (transacylation) of the xenobiotic molecule to protein (fig. 1) (5). During intramolecular rearrangement, the movement of the aglycone moiety away from the 1-C position of the glucuronic acid ring allows opening of the ring, forming an open-chain conjugate with an exposed free aldehyde group.

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to bind covalently to proteins via a second nonenzymic mechanism (glycation) to nucleophilic lysine or N-terminal groups (5, 6). In this case, the entire conjugate, including the glucuronic acid moiety, becomes covalently bound to protein (fig. 1) (7).

The covalent binding of acyl glucuronides to tissue proteins was first demonstrated with plasma proteins, notably albumin (5), and has now been detected in vivo in humans for a number of acyl glucuronide-forming drugs, including beclobric acid (8), clofibric acid (9), diflunisal (10), probenecid (10), salicylic acid (11), tolmetin (12, 13), valproic acid (14), and zomepirac (15).

The role of the acyl glucuronide conjugates, and not the parent compounds, in the formation of adducts with albumin is now well established, based on in vitro studies (5, 7, 8, 11, 14, 15).

Albumin is an important protein involved in the reversible binding and transport of acidic compounds in plasma (16). Although it has been known for some time that many carboxylic acid drugs are transported reversibly bound to albumin in plasma, it was only in recent experiments that the reversible binding of acyl glucuronides to albumin was also demonstrated (17–24). Therefore, in the case of acyl glucuronides, albumin acts not only as a site for reversible binding, but also as a target of acyl glucuronide reactivity; thus becoming covalently modified by these electrophilic metabolites. For some acyl glucuronides, in vitro experiments have shown that the presence of albumin also significantly increases the rate of intramolecular rearrangement and hydrolysis (21, 25–27), and it has been proposed that the reversible interaction between acyl glucuronides and binding sites on the albumin molecule may promote covalent binding and catalysis of acyl glucuronide degradation (17, 26). Albumin may therefore play an important role in the disposition of the reactive acyl glucuronide conjugates by acting as: i) a transporter protein; ii) a potential catalyst for their degradation and, therefore, clearance; and iii) a target for covalent adduct formation.

We have previously demonstrated the reactivity of 1-O-gemfibrozil-β-D-glucuronide in the presence of rat serum albumin (28). In vitro incubations of 1-O-gemfibrozil-β-D-glucuronide with rat serum albumin resulted in degradation of the 1-O-β acyl glucuronide and the associated formation of rearrangement isomers, hydrolysis to reform gemfibrozil, and formation of covalently bound adducts with albumin (28). In vivo studies in rats administered single daily doses of gemfibrozil also revealed formation of gemfibrozil-protein adducts in plasma, kidneys, and liver (28). However, to date, there is no information on the fate of 1-O-gemfibrozil-β-D-glucuronide in humans, particularly with respect to its role in the formation of covalently bound adducts with plasma proteins, notably albumin. The aims of this study, therefore, were to examine the interactions of 1-O-gemfibrozil-β-D-glucuronide with human serum albumin, in particular to: i) examine the potential catalytic role of human serum albumin in the degradation of 1-O-gemfibrozil-β-D-glucuronide; ii) determine whether 1-O-gemfibrozil-β-D-glucuronide is reversibly bound to albumin, and the identity of the binding sites; iii) examine the role of albumin binding sites I and II on intramolecular rearrangement, hydrolysis, and covalent binding of 1-O-gemfibrozil-β-D-glucuronide; and iv) determine whether plasma protein adducts are formed in vivo in humans administered gemfibrozil.

Methods

Gemfibrozil and human serum albumin (fatty acid free) were purchased from Sigma Chemical Company (St. Louis, MO). Lopid tablets were kindly provided by Parke Davis Australia Pty. Ltd. (New South Wales, Australia). 1-O-Gemfibrozil-β-D-glucuronide was biosynthesized as previously described (29). Diazepam was obtained from Roche and oxyphenbutazone from Ciba-Geigy. All other chemicals were of analytical grade, and aqueous solutions were made up in glass-distilled water.

In Vitro Degradation and Covalent Binding to Human Plasma Proteins. 1-O-Gemfibrozil-β-D-glucuronide (250–300 μM) was incubated at 37°C in 1.5-ml aliquots in sealed disposable culture tubes in solutions of: i) 0.1 M phosphate buffer (pH 7.4); ii) 0.1 M phosphate buffer (pH 9.0); iii) drug-free human plasma buffered to pH 7.4 as previously described (20); or iv) human serum albumin (4.0% w/v) in 0.1 M phosphate buffer (pH 7.4). Triplicate incubations were terminated after 0, 0.5, 1, 2, 4, 8, 24, and 48 hr of incubation by placing samples on ice, followed by the immediate addition of 10 μl of orthophosphoric acid. For the samples containing protein, a 1.2 ml aliquot was separated for analysis of covalently bound gemfibrozil-protein adducts. It was frozen in a dry ice/ethanol bath together with the remaining 300 μl aliquot, and stored at −20°C until later analysis.

A further series of incubations with 1-O-gemfibrozil-β-D-glucuronide (250–300 μM) were conducted in a solution of 4.0% (w/v) human serum albumin in 0.1 M phosphate buffer (pH 7.4) at 37°C for 8 hr alone, or in the presence of either diazepam (430 μM)—a site II ligand, oxyphenbutazone (430 μM)—a site I ligand, or gemfibrozil (450 μM). Six replicate incubations were conducted for each incubation condition and were terminated after 8 hr, as described for the longer incubations. Samples were similarly stored frozen until later analysis.

Reversible Binding to Human Serum Albumin. 1-O-Gemfibrozil-β-D-glucuronide was added to a solution of human serum albumin (4.0% w/v) in 0.1 M phosphate buffer (pH 7.4) to give a final concentration of 195 μM. The unbound fraction of 1-O-gemfibrozil-β-D-glucuronide was immediately determined by ultrafiltration using a micropartition filter (Centricon, part no. 4101; Amicon Corporation, Danver MA) centrifuged at 3000g for 15 min at room temperature using an angled rotor. Immediately after centrifugation, 400 μl of ultrafiltrate was sampled and made up to a total volume of 500 μl by the addition of 100 μl of 0.1 M phosphate buffer (pH 7.4). These samples were immediately stabilized by the addition of 60 μl of 0.75 M phosphoric acid, and were stored frozen until later analysis. The unbound fraction was also examined in the presence of diazepam (430 μM), oxyphenbutazone (430 μM), and gemfibrozil (450 μM). All determinations of reversible binding were conducted with six replicates.

Pilot studies had previously established that 1-O-gemfibrozil-β-D-glucuronide was not degraded during the ultra centrifugation procedure and that there was no nonspecific binding to the filter membrane.

In Vivo Formation of Gemfibrozil-Plasma Protein Adducts. Two male patients about to commence clinical treatment with Lopid (500 mg gemfibrozil every 12 hr) volunteered for a pilot study in which trough blood samples (10 ml) were obtained immediately before and at 1, 2, 5, 10, 19, 30, and 90 days after commencement of treatment. Samples were immediately centrifuged, the plasma separated and stabilized by the addition of orthophosphoric acid (30 μl/4 ml of plasma), and stored frozen until later analysis to measure protein adduct concentrations.

Adduct formation during the study was fitted to the following one-compartment pharmacokinetic model assuming zero-order input and first-order elimination:

$$ C_i = C_{i0}[1 - e^{-kt}] $$

where $C_i$ is the concentration of adducts at time $t$ after commencement of dosing, $C_{i0}$ is the concentration of adducts at steady-state, and $k$ is the apparent elimination rate constant.

Analytical Methods. Concentrations of noncovalently bound gemfibrozil, 1-O-gemfibrozil-β-D-glucuronide, and its rearrangement isomers were measured by a direct HPLC method (29). Concentrations of gemfibrozil covalently bound to human serum albumin or plasma proteins were measured by HPLC detection of gemfibrozil after alkaline hydrolysis of the protein adducts, as previously described (28).

Statistical Analysis. Statistical analyses were performed using the nonparametric Mann-Whitney U test (30).
physiological pH and temperature, concentrations of the biosynthetic 1-O-acyl glucuronide declined monoexponentially, with an apparent half-life of 44 hr (fig. 2A). Degradation was significantly increased during incubations in either buffered solution at pH 9.0, drug-free human plasma, or human serum albumin (fig. 2A), with corresponding half-lives for 1-O-gemfibrozil-β-D-glucuronide of 4.1 hr, 5.5 hr, and 8.5 hr, respectively. In all cases, intramolecular rearrangement was the major degradation pathway (fig. 2, B–D). Liberation of parent gemfibrozil was not detectable during incubations in buffer at physiological pH, but small amounts were measured by 48 hr during the more alkaline buffer incubations (fig. 2C). In the presence of human plasma or human serum albumin, liberation of gemfibrozil was greatly increased compared with either pH 7.4 or pH 9.0 buffer incubations (fig. 2C), whereas, the rate of formation of intramolecular rearrangement isomers was also increased, compared with buffer at pH 7.4, but was slightly less than that observed at pH 9.0 (fig. 2B). During both protein-containing incubations, formation of covalently bound gemfibrozil-protein adducts was also evident (fig. 2D) and could not be demonstrated in similar incubations performed with parent gemfibrozil.

The effects of diazepam, oxyphenbutazone, and gemfibrozil on the degradation of 1-O-gemfibrozil-β-D-glucuronide incubated for 8 hr with human serum albumin (pH 7.4, 37°C) are shown in fig. 3A. Diazepam significantly decreased (p < 0.05) the formation of covalently bound adducts and liberation of gemfibrozil to 56% and 63% of control values, respectively. This was accompanied by a significant increase (p < 0.05) in the concentrations of both the biosynthetic 1-O-β acyl glucuronide and its rearrangement isomers to 108% and 105% of control values, respectively. Oxyphenbutazone had no effect on formation of covalently bound adducts (p > 0.05) or the liberation of gemfibrozil (p > 0.05). However, it produced a significant increase (p < 0.05) in intramolecular rearrangement to 109% of control values, with a corresponding decrease (p < 0.05) in the concentrations of the 1-O-β acyl glucuronide to 86% of control values. Gemfibrozil had effects similar to oxyphenbutazone, producing a significant increase (p < 0.05) in intramolecular rearrangement to 117% of control values, with a corresponding decrease (p < 0.05) in the concentrations of 1-O-β acyl glucuronide to 76% of control values, but had no effect on formation of covalently bound adducts (p > 0.05). The effects of gemfibrozil on the hydrolysis of the gemfibrozil glucuronides could not be determined.

1-O-Gemfibrozil-β-D-glucuronide was highly reversibly bound to human serum albumin, with a mean (SD) unbound fraction of 0.028 (0.003) at a total concentration of 195 μM. The effects of diazepam, oxyphenbutazone, and gemfibrozil on the unbound fraction of 1-O-gemfibrozil-β-D-glucuronide are shown in fig. 3B. In the presence of diazepam, the unbound fraction was increased 2.10-fold (p < 0.05). Oxyphenbutazone and gemfibrozil also increased (p < 0.05) unbound fraction 1.24-fold and 2.17-fold, respectively.

The in vivo formation of covalently bound adducts with human plasma proteins is shown in fig. 4. In both patients, adduct concentrations were detectable from the first day of gemfibrozil administration, reaching steady-state concentrations of 5.05 and 8.45 nmol of gemfibrozil covalently bound to proteins per ml of plasma, with corresponding half-lives of 3.0 and 2.5 days, respectively.
Degradation of 1-O-gemfibrozil-β-D-glucuronide and formation of ( ) rearrangement isomers, ( ) gemfibrozil, and ( ) covalently bound adducts after 8-hr incubation at pH 7.4 and 37°C. Unbound fraction of 1-O-gemfibrozil-β-D-glucuronide ( ). Results are shown as mean (±SD) of individual values in the presence of inhibitor, normalized for the corresponding mean control value in the absence of inhibitor (*p < 0.05 vs. control values). Mean (SD) amounts of 1-O-gemfibrozil-β-D-glucuronide, rearrangement isomers, gemfibrozil, and adducts present in control incubations were 51.5% (0.2), 32.8% (0.3), 6.5% (0.1), and 0.204% (0.014) of total gemfibrozil equivalents, respectively. Mean (SD) control fraction unbound was 0.028 (0.003).

Discussion

Consistent with previous reports of acyl glucuronide reactivity (5), the present study demonstrates that, like other acyl glucuronides, 1-O-gemfibrozil-β-D-glucuronide is chemically reactive, undergoing intramolecular rearrangement and hydrolysis in a time and pH-dependent manner. The half-life of 1-O-gemfibrozil-β-D-glucuronide in aqueous solution buffered at physiological pH and temperature (44 hr) is longer than that reported for any other acyl glucuronide studied to date (5), indicating that it may be one of the least chemically reactive acyl glucuronides. Benet et al. (31) proposed that the in vitro stability of acyl glucuronides is related to the degree of substitution of the carbon adjacent to the aglycone carboxylate function. 1-O-Gemfibrozil-β-D-glucuronide is fully substituted at this position, and its low reactivity is consistent with such a proposal.

In the presence of either human plasma or human serum albumin, the half-life of 1-O-gemfibrozil-β-D-glucuronide was significantly decreased, suggesting a catalytic role for human plasma proteins in the degradation of this conjugate. Compared with buffer at physiological pH and temperature, the presence of human plasma proteins increased both the rate of intramolecular rearrangement and hydrolytic regeneration of aglycone (fig. 2, B and C). In addition, in the presence of protein, a third degradation pathway, adduct formation (fig. 2D) was also available. The degree of intramolecular rearrangement and hydrolysis was similar during both human plasma and albumin incubations, suggesting that albumin accounts for most of the catalytic effect of human plasma on the degradation of 1-O-gemfibrozil-β-D-glucuronide. Albumin is known to have esterase-like activity toward esters, amides, and phosphates (32–34). The catalytic effect of albumin on the degradation of 1-O-gemfibrozil-β-D-glucuronide is consistent with an esterase-like activity of albumin toward acyl glucuronides, and has been observed with other acyl glucuronides including, those of oxaprozin (26), fenoprofen (27), and ketoprofen (21, 25, 35). The role of albumin in increasing intramolecular rearrangement of acyl glucuronides has also been reported for oxaprozin glucuronide (26).

The esterase-like activity of albumin toward esters, amides, and phosphates has been associated with the classical site I (warfarin) and site-II (diazepam) binding domains of albumin, as well as other, possibly nonspecific, binding sites (32–34). In this study, oxyphenbutazone, gemfibrozil, and diazepam were used to investigate the involvement of the site I and II albumin binding domains on the degradation of 1-O-gemfibrozil-β-D-glucuronide. Only diazepam produced a statistically significant inhibition of hydrolysis and covalent binding (fig. 3A), suggesting that, for the gemfibrozil glucuronides, reactive amino acid residues at the site II binding domain may be involved in the esterase-like activity of albumin (17) showing that prior covalent modification of tyrosine residues on albumin inhibited the catalysis of oxaprozin glucuronide covalent binding, hydrolysis, and intramolecular rearrangement. Other reactive amino acid residues in the site II binding domain may also be involved in covalent binding of acyl glucuronides, such as lysine-541, lysine-525, and serine-480, which are covalently modified by tolmetin glucuronide in vitro (7). In control incubations, hydrolysis and covalent binding together made up

![Graph](image-url)

**Fig. 3.** Effects of diazepam (Diaz), oxyphenbutazone (Oxy), and gemfibrozil (Gem) on the fate of 1-O-gemfibrozil-β-D-glucuronide incubated in the presence of human serum albumin.

**Fig. 4.** Plasma concentrations of covalently bound gemfibrozil-protein adducts in two patients after commencement of treatment with gemfibrozil (1 g/day po).
∼7% of total gemfibrozil equivalents at 8 hr. In the presence of diazepam, the inhibition of these two pathways by ∼50% largely accounts for the small increase in the contents of 1-O-gemfibrozil-β-D-glucuronide and its rearrangement isomer. The site I ligand oxyphenbutazone had no effect on either the hydrolysis or covalent binding of the gemfibrozil glucuronides (fig. 3A), consistent with the involvement of the site II binding domain in these two degradation pathways. However, oxyphenbutazone produced a statistically significant increase in the formation of the rearrangement isomer, which was reflected in an approximately equal but opposite decrease in the amount of 1-O-gemfibrozil-β-D-glucuronide (fig. 3A). This suggests that site I does not catalyze the intramolecular rearrangement of 1-O-gemfibrozil-β-D-glucuronide. It seems more likely that intramolecular rearrangement is catalyzed at an alternative binding site(s) on albumin, distinct from either the site I or II binding domains. Oxyphenbutazone may increase the albumin-catalyzed intramolecular rearrangement of 1-O-gemfibrozil-β-D-glucuronide in two ways. The reversible binding of oxyphenbutazone with albumin may produce a conformational change, facilitating the interaction of 1-O-gemfibrozil-β-D-glucuronide with the rearrangement catalytic site(s). Alternatively, in the presence of oxyphenbutazone, less 1-O-gemfibrozil-β-D-glucuronide would be reversibly bound to the noncatalytic site I, making more of the conjugate available for binding at the site(s) catalyzing intramolecular rearrangement. Gemfibrozil, which is highly reversibly bound to albumin (1), behaved like oxyphenbutazone, consistent with it being a ligand for the site I binding domain (1).

Alteration of 1-O-gemfibrozil-β-D-glucuronide’s degradation by diazepam, oxyphenbutazone, and gemfibrozil seemed to be directly related to each compound’s ability to inhibit the reversible binding of the conjugate to albumin (fig. 3B). At physiological concentrations of human serum albumin, 1-O-gemfibrozil-β-D-glucuronide was highly reversibly bound to albumin, with an average unbound fraction of 0.028. This high degree of binding to albumin is similar to that previously reported for the acyl glucuronide conjugates of fenoprofen (24), carprofen (18), tolmetin (23), zomepirac (23), and diflunisal (19, 20). The reversible binding of 1-O-gemfibrozil-β-D-glucuronide to albumin seemed to involve at least two different binding sites, because both diazepam and oxyphenbutazone were able to displace the conjugate. The direct relationship between displacement of 1-O-gemfibrozil-β-D-glucuronide and inhibition of the conjugate’s degradation is supported by the observation that oxyphenbutazone, which produced a smaller increase in unbound fraction compared with gemfibrozil (fig. 3B), also produced a smaller change in degradation compared with gemfibrozil (fig. 3A). These observations are consistent with the work of Wells et al. (17), who proposed that the degradation and covalent binding of oxaprozin glucuronide in the presence of albumin was directly related to the reversible binding of the conjugate to albumin.

Reactivity of 1-O-gemfibrozil-β-D-glucuronide was also observed in vivo in two patient volunteers, in whom covalently bound gemfibrozil-protein adducts were detectable in plasma (fig. 4). The half-life of the plasma adducts (2.5–3.0 days) was generally shorter than that reported for other plasma protein adducts in humans (11, 13), all of which are also shorter than the half-life of albumin (~19 days) in humans (36). We may assume that the majority of in vivo plasma protein adducts are formed with albumin and that the volume of distribution of the modified albumin is the same as that of native albumin. If so, then, the varying in vivo half-lives of glucuronide-derived adducts may be a reflection of both the chemical stability of the drug–albumin bond—which may depend on the type of amino acid residue involved and the mechanism of covalent binding (i.e., transacylation or glycation)—and also the effects of covalent modification of albumin on its conformation, which may make it more easily cleared than native albumin. Alternatively, covalent binding may involve proteins other than albumin, and the varying half-lives may be a reflection of the different degradation rates of different proteins.

Benet et al. (31) have tried to predict in vivo adduct formation from in vitro data of acyl glucuronide reactivity. They have shown that, for a series of acyl glucuronide-forming drugs, the amount of in vivo adduct formation in humans was directly related to the plasma area under the curve of acyl glucuronide metabolite (31). In addition, they also demonstrated that, after a single dose of carboxylic acid drug, plasma protein adduct formation normalized for acyl glucuronide area under the curve was directly related to the in vivo degradation rate of the acyl glucuronide in the presence of human serum albumin (31). However, to predict the amount of adduct accumulation over long-term treatment with acyl glucuronide-forming drugs, it will be necessary to also know the half-life of the adducts in vivo. This may be difficult to predict from in vitro reactivity data, because 1-O-gemfibrozil-β-D-glucuronide demonstrates that one of the least chemically reactive acyl glucuronides produces plasma protein adducts with the shortest reported elimination half-lives. It is also unclear whether the extent of plasma protein adduct formation may be predictive of adduct formation with tissue proteins. In a previous in vivo animal study, gemfibrozil-protein adduct formation in plasma was found to correlate with that in kidneys, but not liver (28).

At present the consequences of acyl glucuronide reactivity are unknown. It has been suggested that the formation of albumin adducts may be associated with immune-mediated reactions (5, 37, 38). However, in all of the clinical studies in which plasma protein adducts have been detected, there have been no reports of immune-mediated or other toxicity (8–15). In addition, although in vivo animal studies have demonstrated the generation of antialbumin-adduct antibodies, the albumin adducts injected to sensitize these animals had an epitope density 30 times greater than that seen in vivo in humans (38), or were not formed via an acyl glucuronide intermediate (37). It may be that other adducts, such as those formed with liver or kidney proteins, play an important role in determining toxicity. The interaction of albumin with electrophilic acyl glucuronides may serve a protective function. Albumin may act both as a scavenger of reactive acyl glucuronide metabolites and, in some cases, as a promoter of their degradation; thus protecting other proteins from covalent modification.

**References**


